

Mechanism of action of the group A streptococcal C5a inactivator

(protease/neutrophil receptor/chemotactic factor)

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ABSTRACT Virulent group A streptococci have been found to express a cell-surface factor that has the capability of inactivating complement-derived chemotactic factors. To determine the mechanism of action of this factor, we examined the interaction of purified inactivator with pure C5a chemotaxin. Ligand-receptor binding studies demonstrated that streptococcal chemotactic factor inactivator (SCFI)-treated C5a expressed a greatly reduced ability to bind to receptors of polymorphonuclear leukocytes as compared with native C5a. The inactivation of C5a occurred by a nonstoichiometric and temperature-dependent process. NaDODSO₄/PAGE analysis indicated that SCFI mediated a small decrease in the molecular weight of C5a_{desArg}, and sequencing of the carboxyl terminus of inactivated C5a demonstrated that a six-residue peptide was lost. The release of discrete peptide fragments from denatured bovine serum albumin upon prolonged incubation with SCFI was indicative of endoprotease activity. Although denatured bovine serum albumin was inefficiently cleaved, native bovine serum albumin and other native proteins were highly resistant to SCFI proteolysis; this indicated that activity was specific in nature.

The acute inflammatory response represents an immediate reaction of host tissues and, therefore, the first line of defense to invading microorganisms. Activation of the alternative (antibody-independent) complement system by components of the microbial cell surface serves the host as a recognition event to signal the occurrence of an invasion. This activation is characterized by the formation of fluid-phase C5a and C3a anaphylatoxins, both of which stimulate smooth muscle contraction, increase vascular permeability, and trigger degranulation and release of histamine from mast cells and basophils (1). Furthermore, C5a is a potent stimulant for phagocytic leukocytes, inducing a burst of metabolic activity (2, 3), release of toxic superoxide radicals (4), and release of lysosomal enzymes (5, 6). In the form of a concentration gradient, it also provides a chemotactic signal. C5a is the major chemoattractant generated in serum after activation of complement (7), and in many situations it is responsible for the rapid neutrophilic infiltration at inflammatory loci (8).

There are three major strategies by which the complement-related chemotactic response can be reduced or prevented in host-parasite interactions: (i) the inhibition of complement activation, (ii) secretion of toxins that suppress cellular responsiveness to chemotactic stimuli, and (iii) inactivation of generated chemotaxins. Until recently, evidence for inactivation of complement-derived chemotactic factors has been scant. While bacterial proteases from *Serratia marcescens*, group A streptococci, and *Pseudomonas aeruginosa* have been characterized that have this activity (9, 10), they are nonspecific, readily attacking a variety of protein substrates. In addition, prolonged incubations are required for chemotactic activity to be reduced. The lack of specificity of

these enzymes is further exemplified by the generation of chemotactic fragments from native C5 (9, 10), a phenomenon that would appear counterproductive in terms of phagocyte evasion.

We have previously reported on the ability of virulent group A streptococci to inactivate complement-derived chemotactic activity via an uncharacterized cell-surface molecule that was distinguished from the virulence marker, M protein (11). This factor has since been purified and was found to be an acidic high molecular weight protein antigen (28). We have provisionally designated this antigen the streptococcal chemotactic factor inactivator (SCFI) on the basis of its anti-chemotactic activity. In this report, we describe our investigations into the mechanism by which SCFI inhibits the biologic activity of C5a.

MATERIALS AND METHODS

Isolation of SCFI. SCFI was extracted from M49 T14 group A streptococci by a limited trypsin digestion and was purified by hydrophobic interaction chromatography, anion-exchange chromatography, and gel filtration (28). Hyperimmune antiserum to SCFI was produced by repeated injection of rabbits with the purified protein emulsified in Freund's adjuvant (12), and the content of specific antibody was determined by immunodiffusion and neutralization of SCFI activity.

Preparation of Human C5a and C5a_{desArg}. C5a was generated in zymosan-activated human serum containing 1 M ϵ -aminocaproic acid and it was purified according to the procedure of Fernandez and Hugli (13). C5a_{desArg} was purified by the same method except that serum was activated in the absence of ϵ -aminocaproic acid. Human C5a was radio-labeled with Na¹²⁵I (Amersham) by the solid-phase lactoperoxidase/glucose oxidase method as described (14). ¹²⁵I-labeled C5a_{desArg} for analysis by NaDODSO₄/PAGE analysis was obtained from Upjohn.

Purification of SCFI-Inactivated C5a. C5 (10 nmol) was mixed with 20 μ g of SCFI in phosphate-buffered saline/1% bovine serum albumin, pH 7.4, and incubated for 2 hr at 37°C. The reaction mixture was applied to a 1.0-ml anti-C5a immunoaffinity column previously prepared by conjugating murine anti-C5a IgG with cyanogen bromide-activated Sepharose 4B. After washing with 0.1 M PO₄/0.5 M NaCl, pH 7.8, the bound C5a antigen was subsequently eluted with 30% (vol/vol) acetic acid, pooled, and lyophilized.

Preparation of Human Peripheral Polymorphonuclear Leukocytes (PMNs). Human PMNs were collected by venipuncture of healthy volunteers as described by Boyum (15). After Ficoll-Hypaque centrifugation (16), the PMN-containing layer was subjected to hypotonic lysis in 0.87% NH₄Cl to remove contaminating erythrocytes (17). The PMNs were harvested by centrifugation at 150 \times g for 10 min, washed twice in 0.9% NaCl, and resuspended to the appropriate cell

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Abbreviations: SCFI, streptococcal chemotactic factor inactivator; PMN, polymorphonuclear leukocyte.

density in RPMI (GIBCO) tissue culture medium containing 1% bovine serum albumin.

C5a Binding Assay. Binding of ^{125}I -labeled C5a to PMNs was assessed by mixing 4×10^6 PMNs per ml in RPMI medium/1% bovine serum albumin with ^{125}I -labeled C5a at a final concentration of 1 nM in a 0.1-ml vol. The mixtures were incubated for 15 min at 24°C in 1.5-ml conical polypropylene microfuge tubes followed by centrifugation at 11,000 $\times g$ for 30 sec in a Beckman Microfuge B. The amount of cell-bound ^{125}I -labeled C5a was determined by transferring one-half of the PMN-free supernatant to a separate tube and comparing the γ radioactivity of the paired samples P (pelleted cells + one-half supernatant) and S (one-half supernatant). Cell-bound ^{125}I -labeled C5a was calculated according to the following equation:

$$\% \text{ bound} = \frac{\text{cpm}(S + P) - \text{NS cpm}(S)}{\text{cpm}(S + P)} \times 100.$$

NS, nonspecific binding to the assay tube, was $\text{cpm}(P + S)/\text{cpm}(S)$. Of the total ligand, 30–50% was PMN-associated under these conditions (18).

Radioimmunoassay for Human C5a. Quantitation of C5a antigen was achieved with radioimmunoassay procedures as described (18). Briefly, 30 μl of 1 nM ^{125}I -labeled C5a, 10 μl of sample, and 10 μl of appropriately diluted rabbit C5a antibody were mixed and incubated for 15 min at 24°C. Separation of bound and free ^{125}I -labeled C5a was performed by addition of 50 μl of formalin-fixed protein A-bearing *Staphylococcus aureus* (IgGsorb, The Enzyme Center, Boston, MA). After 15 min of incubation at 24°C, the IgGsorb was sedimented by centrifugation at 11,000 $\times g$ for 30 sec and the samples were processed as described for the PMN binding assay. Standard curves (B/B_0 vs. concentration of antigen) were generated with native C5a (1–100 nM) serving as the competing antigen.

Amino Acid Analysis. For determination of free amino acids, unhydrolyzed samples or 2 nmol of hydrolyzed C5a standard were examined by using a Durrum amino acid analyzer. Hydrolysis of C5a was done in 5.6 M HCl by heating in a vacuum-sealed ampoule at 100°C for 18 hr.

Carboxyl-Terminal Analysis. C5a_{desArg} (1 nmol) or purified SCFI-inactivated C5a in 0.1 M NH₄HCO₃ (pH 8.15) was digested with 0.1 nmol of carboxypeptidase A (phenylmethylsulfonyl fluoride-treated) (Worthington) and 0.1 nmol of carboxypeptidase B (Calbiochem) for 4.5 hr at 37°C (19). These samples were lyophilized and analyzed for free amino acids in the absence of hydrolysis as described above.

NaDODSO₄/PAGE. Discontinuous polyacrylamide gel electrophoresis was carried out under reducing conditions by a modification of the procedure of Laemmli (20). For electrophoresis of ^{125}I -labeled C5a_{desArg}, a separation gel was used consisting of an exponential gradient of 15–20% acrylamide (12–16% glycerol) and radiolabeled protein visualized by autoradiography with Kodak XAR-5 x-ray film. Denatured bovine serum albumin was prepared by a modification of the alkaline urea method of Anson (21). Bovine serum albumin (50 μg) in H₂O was first incubated with 50 mM dithiothreitol for 10 min at room temperature, then the protein was alkalinized with NaOH (0.08 M final NaOH concentration). Crystalline urea was added to 7.3 M, and the mixture was incubated at room temperature for 1 hr. The denatured bovine serum albumin was diluted with an equal volume of 0.062 M Tris-HCl (pH 7.4) and dialyzed overnight against the same buffer. A 5–15% acrylamide gradient was used for electrophoresis of bovine serum albumin (Sigma), and silver staining was by the Oakley method (22).

RESULTS

Inactivation of ^{125}I -Labeled C5a by SCFI. While SCFI could clearly inhibit the chemotactic activity of zymosan-activated human serum (11), it remained to be determined whether purified C5a could be similarly inactivated. To examine this, the concentration-dependent effect of SCFI on the PMN receptor binding and antigenic properties of ^{125}I -labeled C5a was assessed. SCFI, ranging in concentration from 0 to 625 ng/ml, was incubated with 5 nM ^{125}I -labeled C5a (41 ng/ml) for 60 min at 37°C, and the ability of treated ligand to bind PMNs was measured. As shown in Fig. 1, specific PMN binding of ^{125}I -labeled C5a was completely inhibited by prior incubation of the ligand with SCFI (10 ng/ml), and as little as 0.07 ng of SCFI per ml produced a significant binding inhibition. The possibility that this effect was due to the complete destruction of C5a by either SCFI or enzymatic contaminants of the SCFI preparation was highly unlikely because the antigenicity of the ^{125}I -labeled C5a remained unaltered (Fig. 1).

Comparison of Receptor Binding Affinities of SCFI-treated C5a and Untreated C5a. Competitive PMN binding experiments were performed to show that SCFI acts on unlabeled C5a as well as on ^{125}I -labeled C5a, and to provide an estimate of the decreased ligand receptor binding affinity of the inactivated C5a. As shown in Fig. 2, a comparison of native and immunoaffinity-purified SCFI-treated C5a demonstrated that the affinity of SCFI-treated C5a for PMN receptors was considerably less than that of the native ligand as determined by competition for binding sites with ^{125}I -labeled C5a. Comparison of the concentrations of C5a and SCFI-treated C5a that produced a 10% inhibition of ^{125}I -labeled C5a binding suggested that SCFI treatment reduced the apparent affinity of C5a by a factor of ≈ 150 . The residual binding activity of SCFI-treated C5a, possibly due to a weaker binding affinity, could also be accounted for by the presence of a small amount ($\approx 0.7\%$) of native C5a.

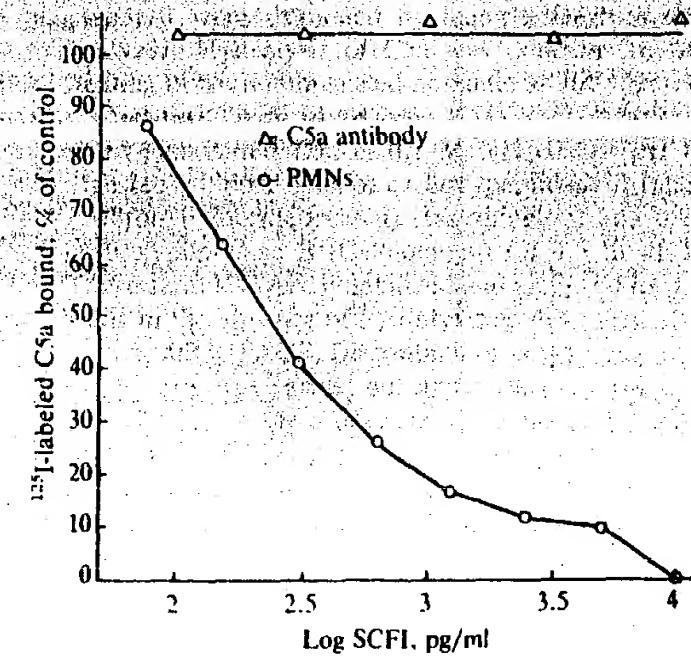


FIG. 1. Dose response for C5a inactivation by SCFI. Purified SCFI (0.076–10 ng/ml) was incubated with 5 nM ^{125}I -labeled C5a for 60 min at 37°C. ^{125}I -labeled C5a was incubated in the absence of SCFI as a negative control. PMN binding was assessed by incubating each mixture with 4×10^6 PMNs for 15 min at 24°C, followed by centrifugation to pellet the cells. One-half of the supernatant was transferred to a separate tube and the radioactive counts in the paired samples (pellet + one-half supernatant and one-half supernatant) were compared. Antigenicity measurements were obtained by radioimmunoassay. Values are the mean of duplicate determinations.

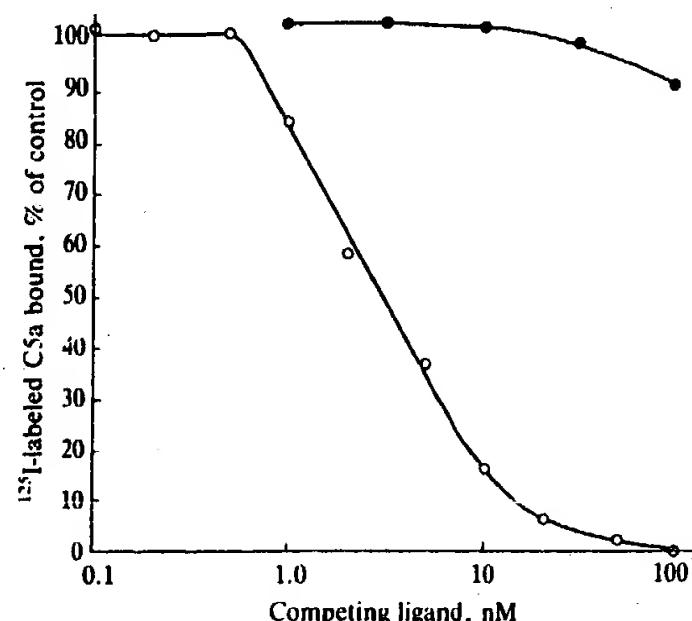


FIG. 2. Comparison of ligand-receptor affinity by competitive PMN binding. C5a or immunoaffinity-purified SCFI-treated C5a (10–1000 nM) was added to an equal volume of 10 nM 125 I-labeled C5a. These samples were diluted 1:10 with PMNs (3×10^6 per ml) and binding of 125 I-labeled C5a to the PMNs determined. Values are the mean of duplicate determinations. Open circles indicate untreated C5a and solid circles indicate SCFI-treated C5a.

Temperature Dependency of C5a Inactivation. Fig. 1 suggested that SCFI was acting as an enzyme. To support this hypothesis, we examined the temperature dependency of the rate of C5a inactivation. The rate of reaction was defined at 4°C, 24°C, and 37°C; each reaction terminated by addition of SCFI antiserum to neutralize SCFI activity. The results in Fig. 3 demonstrate that the rate of inactivation of 125 I-labeled C5a was clearly dependent on the incubation temperature. After 60 min, there was complete inactivation only at 37°C, with ~90% inactivation at 24°C and 50% inactivation at 4°C.

Proteolytic Cleavage of C5a by SCFI. NaDODSO₄/PAGE analysis was performed to determine whether inactivation of 125 I-labeled C5a_{desArg} by SCFI was accompanied by a decrease in molecular weight, as would be expected from enzymatic cleavage of the molecule. As shown in Fig. 4 (lane

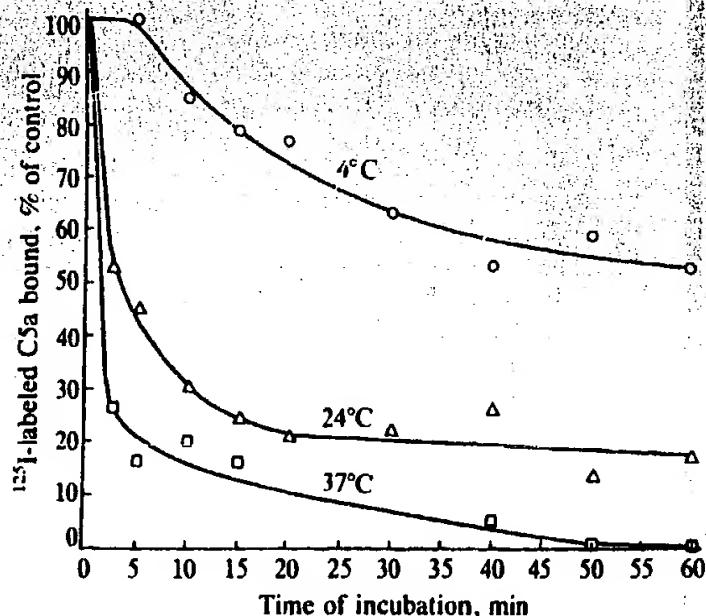


FIG. 3. Temperature dependency of the rate of C5a inactivation. 125 I-labeled C5a (5 nM) was incubated with SCFI (10 ng/ml) at 4°C, 24°C, or 37°C. Aliquots were removed at intervals and incubated for 15 min at 24°C with rabbit anti-SCFI antibody to terminate the reaction, and PMN binding was assessed. Values are the mean of duplicate determinations.



FIG. 4. NaDODSO₄/PAGE of SCFI-treated 125 I-labeled C5a. 125 I-labeled C5a_{desArg} (10 μ l) (2.8×10^5 cpm/ml) was incubated with (lane B) or without (lanes A) 125 ng of SCFI for 60 min at 37°C. Samples were mixed with sample buffer containing 3.3% NaDODSO₄ and 2.5% 2-mercaptoethanol, and then boiled for 2 min. Discontinuous NaDODSO₄/PAGE was performed by using a 15–20% exponential gradient, and proteins were visualized by autoradiography.

B), 125 I-labeled C5a_{desArg} incubated with SCFI exhibited a significantly greater electrophoretic mobility than untreated 125 I-labeled C5a_{desArg} (lanes A). This difference in mobility was small, corresponding to a M_r of ≈ 500 , suggesting that SCFI mediates the cleavage of 125 I-labeled C5a_{desArg} near the amino or carboxyl terminus.

To locate the peptide bond in C5a that is cleaved by SCFI, the carboxyl-terminal sequence of immunoaffinity-purified SCFI-inactivated C5a was compared with that of native C5a_{desArg}. Under partial digestion conditions, the relative amount of each amino acid released by carboxypeptidases A and B depends on its distance from the COOH terminus of the peptide in question (19). Because the sequence of C5a is known, this approach will unequivocally identify the COOH-terminal residue. The results presented in Table 1 demonstrate that SCFI-inactivated C5a lacks the end sequence -Asp⁶⁹-Met⁷⁰-Gin⁷¹-Leu⁷²-Gly⁷³-Arg⁷⁴ (23), and therefore C5a cleavage occurs between Lys⁶⁸ and Asp⁶⁹. The detection of the amino acids phenylalanine, tyrosine, and threonine is not compatible with the known sequence of C5a. The aberrant release of phenylalanine and tyrosine could in part be the result of contamination of the carboxypeptidases with a chymotryptic-like activity. In addition, it is necessary to postulate additional contaminants in the SCFI-C5a_{desArg} mixture in order to account for the release of a relatively high concentration of threonine. Alternatively, the immunoaffinity-purified C5a_{desArg} could have contained a small fraction of partially degraded molecules. The relative yield of these amino acids does not allow us to conclude which component was contaminated; nevertheless, this does not detract from the conclusion that one COOH terminus predominated in each preparation.

Endoprotease Activity of SCFI. The possibility that an exopeptidase activity of SCFI participated in the cleavage of C5a was examined. A reaction mixture containing 2 nmol of C5a and 4 μ g of SCFI was tested for the appearance of free amino acids by automated amino acid analysis. SCFI was analyzed separately as a control for background amino acids, and acid-hydrolyzed C5a served as the amino acid standard. No amino acids above control levels were detected in this mixture, suggesting that C5a was not degraded by SCFI through an exopeptidase function. In addition, a small basic peak was detected by the amino acid analyzer that did not correspond with the C5a amino acid standards; this most likely represented a short peptide derived from the C5a.

Table 1. Carboxyl-terminal analysis of SCFI-inactivated C5a

Amino acid	C5a _{desArg} , nmol	SCFI-inactivated C5a*, nmol
Gly	1.06	0.11
Leu	0.86	0.06
Glx	0.85	0.02
Met	0.37	0.00
Asx	0.32	0.00
Lys	0.32	1.00
His	0.28	1.12
Ser	0.00	0.67
Ile	0.13	0.19
Phe	0.12	0.26
Tyr	0.15	0.78
Thr	0.06	0.36

Known sequence:					
65	68	71	74		
- Asn - Ile - Ser - His - Lys - Asp - Met - Gln - Leu - Gly - (Arg) - COOH					
C5a _{desArg} , nmol	0.28	0.32	0.32	0.37	0.85
SCFI-C5a, nmol	0.67	1.12	1.00		

*One nanomole of carboxypeptidase-digested C5a_{desArg} or SCFI-inactivated C5a was analyzed. SCFI inactivation was assessed by the reduction in ligand-receptor binding affinity.

These observations together support the hypothesis that the shortening of C5a occurs through an endoproteolytic cleavage.

Substrate Specificity of SCFI. The specificity of SCFI was examined in terms of its ability to cleave a variety of proteins unrelated to C5a. Potential substrates were selected on the basis of containing one or more lysyl-aspartyl peptide bonds, and digestion conditions were similar to those providing for complete inactivation of C5a PMN binding, except for the use of extended incubation periods. The effect of SCFI on native and denatured bovine serum albumin was representative of these analyses. It was found that cleavage of the native protein was minimal, even after 48 hr at 37°C (Fig. 5A). After urea denaturation, however, the bovine serum albumin was cleaved to a greater extent, with lower molecular products

appearing within 1 hr (Fig. 5B). The appearance of discrete peptide fragments on prolonged incubation is characteristic of endoproteolytic cleavage. Other proteins tested for SCFI sensitivity were highly resistant in the native state, including human serum albumin, ovalbumin, soybean trypsin inhibitor, carbonic anhydrase, α -lactalbumin, myosin, and cytochrome *c*. As with bovine serum albumin, several showed a slight increase in lability to SCFI after prior denaturation with urea.

DISCUSSION

The truncation of the carboxyl end of C5a has been shown previously to reduce the ability of the molecule to stimulate the leukocyte functions of chemotaxis and exocytosis (3, 24), which are involved in the development of inflammation and a microbicidal environment in the vicinity of complement-activating microorganisms. This loss of C5a functional activity is due at least in part to a reduced binding affinity for the C5a receptors of PMNs (25). After complement activation in serum, the C5a that is generated is immediately attacked by an endogenous carboxypeptidase B-like enzyme (carboxypeptidase N) that specifically removes the COOH-terminal arginine residue to form the derivative, C5a_{desArg} (26). This results in the essentially complete loss of intrinsic spasmodic and vascular permeability-increasing properties (26) as well as a decrease by a factor of 10–20 in the ability to promote leukocyte functions (25). The complete conversion of C5a to C5a_{desArg} is consistent on a quantitative basis with the C5-related chemotactic activity found in zymosan-activated serum (25), and this conversion probably serves to limit the tissue-destructive effects of C5a-induced inflammation (27). The sequential removal of five amino acids from the carboxyl-terminal end of C5a with yeast carboxypeptidase Y to form the analogue C5a-(1–69) results in the complete loss of functional activity and a parallel, although incomplete, reduction in binding affinity (25). The significant residual binding capacity of C5a-(1–69) has led to the proposal that the carboxyl-terminal region provides an essential receptor recognition function auxiliary to the internal portion of the ligand, as well as an "activation" site required for the stimulation of receptor-mediated functions (25).

The results of the present study show that SCFI is a protease that attacks the COOH-terminal region of C5a at the carboxyl side of a lysine residue to produce a molecule that lacks the capacity for functional interaction with PMN

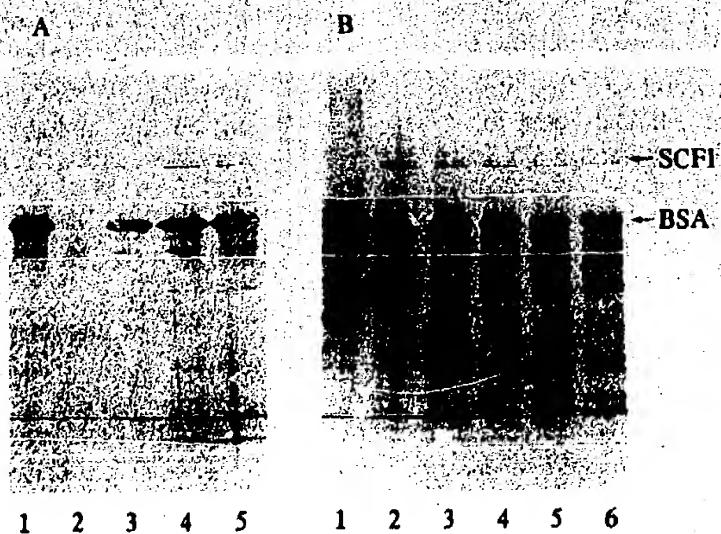


FIG. 5. Proteolytic activity of SCFI with bovine serum albumin as the substrate. Bovine serum albumin (80 μ g/ml) (A) or urea-denatured bovine serum albumin (B) was incubated in 0.0625 M Tris-HCl (pH 7.4) with SCFI (20 μ g/ml) at 37°C. Aliquots (10 μ l) were removed at various time points, boiled with sample buffer, and analyzed by NaDODSO₄/PAGE. The gel consisted of a 5–15% acrylamide gradient, and proteins were stained by the silver technique. BSA, denatured bovine serum albumin at 48 hr (lanes 1); SCFI at 48 hr (lanes 2); bovine serum albumin at 11 hr (lane 3 in A), 24 hr (lane 4 in A), 48 hr (lane 5 in A). Denatured bovine serum albumin at 11 hr (lane 3 in B), 8 hr (lane 4 in B), 24 hr (lane 5 in B), 48 hr (lane 6 in B).